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14. ABSTRACT

Improved molecular diagnostic methods that can classify tumors and predict their response to therapy have enormous potential to improve the effectiveness breast cancer treatments. The overall goal of this project was to develop a novel molecular diagnostic method, termed SH2 profiling, that can classify cell samples based on their global protein tyrosine phosphorylation state. The first aim was to use an existing SH2 profiling method, based on far-Western blotting, to analyze fresh surgical breast cancer samples. The second aim was to develop a more high-throughput quantitative reversed-phase SH2 profiling format, and test its usefulness in classifying breast cancer samples. The third aim was to develop histochemical SH2 profiling methods that can be used to analyze archived, formalin-fixed tissue sections, and perform pilot retrospective studies to determine whether SH2 binding patterns have potential prognostic value. Over the course of this study we have made great progress in developing a robust, quantitative, high-throughput SH2 profiling method. We have constructed a nearly complete set of human SH2 domain probes. We have used these tools to profile 20 human breast cancer surgical specimens. We find that SH2 profiles differ among different breast cancer samples, and provide information beyond that provided by standard clinical-pathological staging. Thus we have demonstrated the feasibility of SH2 profiling as a novel molecular diagnostic tool for classifying cancer and potentially predicting clinical outcomes.

15. SUBJECT TERMS
Molecular diagnostics, prognostic classification of tumors, phosphotyrosine. SH2 domains, signal transduction, protein microarrays, histochemical analysis

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INTRODUCTION

The focus of this study is to test whether a novel molecular diagnostic approach, which we term SH2 profiling, can serve as a useful prognostic tool to classify breast cancer. SH2 domains are small protein modules that bind specifically to tyrosine-phosphorylated peptides. These domains play an important role in normal signal transduction, mediating the formation of multiprotein complexes in response to changes in tyrosine phosphorylation (Liu et al., 2006; Machida and Mayer, 2005). There are ~120 SH2 domains in the human genome, and different SH2 domains recognize different tyrosine phosphorylated proteins. SH2 profiling is a method in which a battery SH2 domain probes is used to provide a snapshot of the global state of tyrosine phosphorylation in a cell sample (Dierck et al., 2006; Machida et al., 2003; Nollau and Mayer, 2001). Different breast cancers are likely to have different patterns of tyrosine phosphorylation, reflecting differences in the signal transduction pathways active in the tumor, and thus SH2 profiling has the potential to provide a biologically relevant means to classify these tumors. In this project we proposed to test different formats of SH2 profiling (far-western blotting, reverse-phase arrays, histochemical analysis of fixed tumor sections) using breast cancer samples to test the feasibility of these approaches and their potential utility as prognostic tools.

BODY

Task1: Perform SH2 profiling on fresh surgical samples of invasive carcinoma of the breast. Correlate SH2 profile data with standard pathologic criteria and other known molecular markers.

Because of unforeseen difficulties in reconciling the human subjects research protocol approved by the UCHC Institutional Review Board with the requirements of the US Army Human Subjects Research Review Board, human subjects approval for this project was not received until August 2004. Since approval, we have been able to obtain a limited number of surgical samples from the University of Connecticut Health Center and Hartford Hospital. Obtaining adequate numbers of samples from UCHC proved to be quite difficult, due to relatively small numbers of breast cancer patients and turnover among surgical staff. However, we have to date performed reverse-phase SH2 profiling and 1D far-western SH2 profiling on a total of 8 different tumors obtained directly for this project (for some tumors different histologically distinguishable regions of the same tumor were analyzed separately). During the 2005-2006 funding period, we also established a collaboration with Dr. Kevin Claffey at UCHC, who provided us with ~30 frozen breast cancer samples obtained in the course of his study of anti-tumor immunity. We have now profiled 12 of these samples, both by reverse-phase array and subsequent 1D far-western based SH2 profiling. 1D far-western profiling results are described below (Task 2c)

The reverse-phase far-Western array method (described in detail in Task 2, below) was the primary method used to analyze these breast cancer tumor samples. Indeed, we have now come to appreciate that the reverse-phase array is a very powerful screening approach to quickly identify tumors and probes of interest, which can then be analyzed in more detail by 1D far-Western blotting. Thus one of the most important outcomes of this study was the development of a standard approach for SH2 profiling of tumor cells comprising initial screening by reverse-phase array, followed by in-depth analysis by 1D far-Western using a more limited number of samples and probes. This is a highly efficient approach that obtains the maximum information from limited clinical samples and resources.

We have not attempted to correlate the resulting SH2 profiling patterns with clinical outcomes due to the low number of samples. However, as detailed below in our 2004-2005 Annual Report, we have noted in at least one case that the presence of binding sites for a particular SH2 domain (Eat2) correlates with the presence of infiltrating lymphocytes. Also please see Task 2c below, where we have compared SH2 binding profiles with standard clinicopathological criteria such as Her2/Neu amplification and ER/PR status. From these pilot studies it is clear that SH2 profiling provides information beyond what is currently possible using standard pathological

analysis, and thus has the potential to provide novel information that can be used to classify tumors and make predictions regarding clinical outcomes.

Task 2: Develop reverse-phase protein microarray assay to quantitatively analyze SH2 binding profiles of patient tumor samples

a) Test approach using macroarrays of lysates of cell lines (months 1-6)
b) Test miniaturization of assay to microarray format—detection by ECL (months 7-12)
c) Apply to tumor samples of Aim 1 (years 2 and 3)
d) Test fluorescent SH2 labeling and detection (year 2)

Task 2a: Over the entire funding period we devoted a great deal of effort toward developing and validating a robust, reproducible, and quantitative reverse-phase (RP) array platform that can be used to generate SH2 profiling data from complex protein mixtures (cell or tissue lysates) as well as purified phosphoproteins. This assay platform (which we term the rosette RP assay) was described in detail in the 2004-2005 Annual Report. Briefly, protein samples (less than 1 microgram whole cell lysate) are replica-spotted on nitrocellulose membranes in register with the wells of a 96-well blotting apparatus, and then each well is incubated with a different SH2 domain probe. After washing, bound probe is detected by enhanced chemiluminescence (ECL) and quantified by image analysis of scanned X-ray films. Thus the relative binding of many SH2 domains can be quantified for each sample. Reproducibility and reliability of the rosette RP array system were also validated; we have found that inter-spot and inter-assay coefficients of variation range from 8.5% to 42.5% (See 2004-2005 Annual Report). We are confident that if the assay were moved to a true microarray format with robotic spotting and direct digital detection of bound probe, these already modest spotting and detection errors are likely to decrease substantially. We are now preparing a manuscript describing development and validation of the SH2 rosette array and we expect to submit it in the fall of 2006.

Over the past year additional progress has been made in generating a complete set of human SH2 domain probes. At this point we have generated GST fusion constructs for 116 of the total of 120 SH2 domains that have been identified in the human genome (Liu et al., 2006). Thus over the period of this project, we have been able to construct a nearly comprehensive set of probes to allow truly global profiling of the SH2 binding sites in breast cancer tumor samples. This is a major accomplishment and sets the stage for future comprehensive profiling of large sets of tumor samples.

To test which SH2 domain probes provide the strongest signal and lowest background, we used a comprehensive set of 96 GST-SH2 domain probes to screen lysates of two breast cancer cell lines, MDA-MB231 and MDA-MB435, as well as two glioma cell lines, LN229 and U373. Cell lysates were prepared in the presence or absence of pervanadate, a potent tyrosine phosphatase inhibitor. In the absence of pervanadate, endogenous phosphatases very rapidly dephosphorylate tyrosine-phosphorylated proteins in cell lysates. Thus any signal obtained from lysates prepared without vanadate is likely due to nonspecific background binding (i.e. binding is not phosphotyrosine-dependent). In this experiment, a mixture of lysates from tissue culture cell lines briefly treated with vanadate in the culture medium (to enhance tyrosine phosphorylation) or its absence, and then lysed in the presence or absence of pervanadate, were used as positive and negative controls, respectively. 96 GST-SH2 domain probes (including GST alone as a negative control) were incubated with arrayed cell lysates using the rosette RP assay format. Results are shown in Figs. 1A and 1B. As can be seen, most SH2 domain probes demonstrated a positive binding signal for lysates prepared with vanadate, above the background observed in the “vanadateless” lysates.

In Fig. 2, the ratio of the signal for lysates prepared in the presence of vanadate vs. those prepared in its absence is shown, both for the control cell lines and the experimental tumor cell lines (in this case, the combined total

signal for all control or tumor cell samples is shown). More than 50% of the GST-SH2 domain probes showed specific binding to tumor cell and/or positive control lysates compared to negative controls (without vanadate), where specific binding is defined as a greater than two-fold higher signal for lysates prepared with vanadate than for the same lysates in the absence of vanadate. Thus this experiment served to identify those probes that are highly likely to provide useful quantitative binding information for SH2 profiling of breast cancer tissue samples. Most of the SH2 domain probes that had relatively poor signal-to-noise ratios were relatively insoluble when prepared in bacteria, and gave relatively poor protein yields (data not shown). From this, we conclude that new protein constructs or modified bacterial expression protocols will be required to yield better quality probes for these SH2 domains.

Task 2b: We did not pursue the miniaturization of this assay to a microarray (nitrocellulose-coated slide) format because we found that the throughput of the newly established rosette RP format is sufficient for the number of clinical samples now available. Furthermore, the minimal sample requirements of the rosette RP assay (significantly less than 100 micrograms total cell lysate required for screening with the complete set of SH2 domain probes) were compatible with the limited amounts of protein obtained from clinical samples. The macro-array format can also more easily accommodate large numbers of different SH2 probes (up to 96 at one time), whereas a slide-based microarray would require a separate array slide for each probe, leading to lower throughput and reliability, and much higher cost. Thus we felt that the effort to establish the microarray format was not justified at this time. However the rosette RP array experiments provided valuable information regarding sample preparation, blocking, binding and washing conditions, and data analysis that could easily be applied to the microarray format in the future if such a format is deemed advantageous.

Task 2c: In the 2004-2005 Annual report, we described SH2 profiling results for tissue lysates from eight surgical breast cancer samples, plus two adjacent normal samples. We showed that different SH2 domains varied widely in their binding patterns to tumor lysates, and more importantly different breast cancer samples exhibited different patterns of SH2 binding. Background-corrected quantitative binding data were then analyzed using a hierarchical clustering algorithm (Cluster v2.11 (Eisen et al., 1998) with "average linkage clustering" settings). The result was visualized as a "heat map" using Java TreeView (Saldanha, 2004). Clustering and bioinformatics studies were done in collaboration with Dr. Dong-Guk Shin and colleagues in the Department of Computer Engineering at UConn-Storrs.

We have now extended our profiling studies to 12 new tumor samples obtained via collaboration with Dr. Kevin Claffey. In each case, tumor tissue was precisely cored from frozen tissue sections (based on histochemical staining of adjacent sections), ensuring that the SH2 profiles are representative of actual tumor cells, and not associated normal tissues and stroma. This approach is likely to increase substantially the reliability of SH2 profiling data from tumor samples. The rosette RP profiling results for these samples are shown in Figs. 3A and 3B. As can be seen, some SH2 domain probes gave strong signals for positive controls but not for breast cancer samples (e.g. ShcA, Fig. 3B), indicating low levels of tyrosine phosphorylated binding sites for those SH2 domains in the tumor samples. On the other hand, other probes gave relatively strong signals for breast cancer samples and weak signals for positive controls (e.g. Aps, Fig. 3B). In this case, signals from tumor samples are likely due to nonspecific background binding, as 10X more lysate was spotted for tumor samples compared to controls. The SH2 domains most likely to provide useful SH2 profiling results (favorable signal-to-noise ratio) are those that give relatively weak but detectable signals for tumor cell lysates and strong signals for the positive control.

Twenty-four SH2 probes with these properties were chosen for further validation by comparing the signal obtained from membranes before and after treatment with PTP-1B, a tyrosine phosphatase that removes phosphotyrosine from cell proteins, thereby destroying specific SH2 binding sites. As shown in Fig. 4, 13 of the 24 SH2 domain probes displayed significantly stronger signals for the untreated membranes compared to the

phosphatase-treated ones. These SH2 domains were then used to probe tumor cell lysates using the 1-D far-Western approach to better visualize SH2 domain binding sites in tumor cell lysates. In this approach, cell lysates are separated by SDS-polyacrylamide gel electrophoresis and transferred to membranes, and probed with labeled GST-SH2 domain fusion proteins. Identical membranes were also probed with anti-phosphotyrosine antibody (anti-pTyr) to reveal the overall quantitative and qualitative patterns of tyrosine phosphorylation in the various breast cancer specimens, and anti-Her2/Neu and anti-EGFR, to identify those samples with amplified EGFR family receptors and to visualize the position of the receptor.

Representative Western and far-Western results are shown in Fig. 5. A number of important conclusions can be drawn from such data. First, individual SH2 domain probes provide much more information than overall tyrosine phosphorylation levels or Her2/Neu status. For example, the phosphatidylinositol 3-kinase (PI3K) SH2 probe specifically binds to proteins present in only a subset of Her2/Neu positive tumors (lanes 9, 10, 16), and furthermore in one of these samples (lane 10), a highly specific protein band of ~110 kDa is identified. Thus PI3K binding sites can potentially be used to sub-classify Her2/Neu positive tumors, perhaps identifying classes that would respond differentially to Her2/Neu-directed therapies. Interestingly, these three samples all show increased EGFR levels (Table 1). It is also of interest that the two tumors shown in lanes 15 and 16 have rather similar overall tyrosine phosphorylation patterns and both exhibit increased Her2/Neu, but can be clearly distinguished on the basis of binding of the PI3K and Vav2 SH2 domains. Finally, the Eat2 SH2 binds strongly and specifically to two bands in lysates of the tumors in lanes 15 and 16, but not to other tumor samples or cell lines. We previously showed in the 2004-2005 Annual Report that Eat2 binding sites correlated with the presence of inflammatory cells in the tumor. Microscopic examination of the tumors corresponding to the Eat2 positive lanes (tumors 52 and 54) showed extensive inflammatory infiltration, consistent with our previous observation (data not shown).

lane	sample ID	pTyr	SH2	EGFR	Erb2
1	LN229	+	Arg		+
2	U373	+		+	+
3	MDA-MB231	+	Arg	+	
4	MDA-MB435	++	Arg,Abl,Crk		
5	Breast Ca #20				+
6	Breast Ca #25				
7	Breast Ca #29	+	Arg.Crk		+
8	Breast Ca #30				+
9	Breast Ca #31	++	Arg,Vav2,PI3K,Sck,Grb2	++	++
10	Breast Ca #34	++	Arg,Vav2,PI3K,Sck,Grb2	++	+++
11	Breast Ca #36	+			+
12	Breast Ca #40		Vav2		++
13	Breast Ca #42		Vav2		+
14	Breast Ca #45				
15	Breast Ca #52	+	Arg,Eat2,Abl,Grb2,Gap,Crk		++
16	Breast Ca #54	++	Arg,Vav2,Eat2.PI3K,Sck,Grb2	++	+++

Table 1. Summary of SH2 profiling results and EGFR and Her2/Neu status for cell lines and clinical samples. Lane numbers correspond to lanes in Fig. 5. pTyr = overall level of tyrosine phosphorylation from anti-pTyr immunoblotting. EGFR, ErbB2 = level of EGFR or Her2/Neu detected by immunoblotting with specific antibodies. SH2 = SH2 probes that exhibit strong binding for each sample.

The results of this set of experiments are summarized in detail in Tables 1 and 2. As can be seen, SH2 profiling results do not correlate well with overall phosphotyrosine, EGFR family amplification, or standard pathological classification (TNM classification). Thus there is great potential for quantitative SH2 binding results to provide novel information that could be used for more detailed classification and prediction of clinical outcomes. Thus a major overall aim of this study has been achieved, setting the stage for larger-scale prospective and retrospective studies to identify clinically useful SH2 binding patterns that correlate with relevant clinical outcomes for breast cancer patients.

lane	tumorID	Diagnosis	LN	ER	PR	HER2	HERtest	TNM
5	20	inf ductal ca	neg	pos	pos	neg	neg	pT1b, pN0, pMX
6	25	inf ductal ca	pos	neg	neg	neg	neg	pT2, pN1mi, MX
7	29	inf ductal ca, lymphovascular invasion	neg	pos	pos	neg	neg	pT2 pN0 pMX
8	30	inf mammary ca	neg	neg	neg	neg	neg	pT2 pN0(i-) pMX
9	31	extensive inf ductal ca in-situ	pos	neg	neg	pos	pos	T1mic, N1, MX
10	34	inf ductal ca	neg	neg/pos	neg	pos	pos	T2, N0, MX
11	36	inf ductal ca	neg	neg	neg	neg	neg	N/A
12	40	inf ductal ca	pos	pos	pos	neg	neg	pT2 pN1a pMX
13	42	inf mammary ca	pos	pos	pos	neg	neg	pT1c,pN1a,MX
14	45	invasive ca w/ ductal and lobular features	pos	pos	pos	neg	neg	pT1c,pN1mi,MX
15	52	inf ductal ca	neg	neg	neg	neg	neg	T1c,N0,Mx
16	54	inf ductal ca	pos	neg	neg	pos	pos	T2, N1

Table 2. Clinical pathological criteria for breast cancer samples analyzed in Figs. 3-5. Lane numbers correspond to those in Fig. 5. LN = lymph node status. ER = estrogen receptor status. PR = progesterone receptor status. HER2, HERtest = two clinical tests for Her2/Neu amplification. TNM = standard TNM classification, using the current American Joint Committee on Cancer (AJCC) staging system.

Task 2d: We have not actively pursued the development of fluorescent SH2 probes, because such probes are unnecessary for the current rosette RP assay format. Since we decided it was not necessary to pursue miniaturization of the assay to a microarray format at this time (Task 2b, above), this sub-aim became superfluous. However, we have laid the groundwork for the facile development of fluorescent SH2 domain probes in the future should it become advantageous. First, we exploited a system in which GST-SH2 fusion proteins are expressed with a C-terminal “avitag”, which directs enzymatic biotinylation by the BirA product in *E. coli* (O'Callaghan et al., 1999). Each SH2 probe is thus tagged with a single biotin moiety, eliminating the disadvantages of heterogeneous multiple biotinylation seen when chemical biotinylation is performed. These singly-biotinylated probes could easily be stoichiometrically labeled using fluorophore-conjugated streptavidin for detection of binding in microarray formats using standard fluorescent array readers. Second, we have entered into a collaboration with Dr. Xudong Yao of the Department of Chemistry at the University of Connecticut Storrs campus. Dr. Yao is an organic chemist who is developing novel covalent glutathione analogs that can be used to stoichiometrically label GST fusion proteins via the glutathione binding site of GST. If this approach is successful, such covalent tags could be easily conjugated to a variety of functional groups, including fluorescent dyes.

Task 3: Establish conditions for using SH2 domains to probe sections of paraffin-embedded tumor samples. Perform retrospective study using archived tumors to test if positive staining with specific SH2 probes correlates with recurrence or survival.

a) Establish histochemical staining method for detection and quantitation of binding of SH2 domains to sections of tumors (year 1)

b) Analyze ~100 archived samples per year with at least 5 SH2 domain probes (ongoing; aim is to analyze at least 200 by end of year 2)
c) Establish database with pathological criteria, patient outcomes; SH2 binding data when available (months 1-6)
d) Correlate SH2 binding with standard pathological criteria (months 6-24)
e) Correlate SH2 binding with patient outcomes (recurrence, mortality) (years 2 and 3)

Work on Task 3 was initially delayed by lack of approval by the HSRRB to perform research on human anatomic samples. Once approval was obtained we performed pilot experiments to better define optimal conditions for histochemical analysis of tissue sections with SH2 domain probes, as outlined in the 2003-2004 Annual Report. Over the final two years of the project, this aim was not been further pursued. The major reason is that we felt that it was more important to devote the limited manpower available to the project to the development of the high-throughput reverse phase far western array, and to generate new SH2 domain probes, as outlined above in tasks 1 and 2. Our pilot experiments for Task 3 in year 1 of the project convinced us that it would take considerable effort to optimize binding conditions for each SH2 domain probe, and furthermore that the throughput of the histochemical assay would be relatively low because a different section would be needed for each SH2 domain (up to a potential total of 120 sections per tumor to screen all SH2 domain probes). It would also be difficult to normalize binding signals for different SH2 domains to provide quantitative outputs that could be used for hierarchical clustering. We felt that before going on to large-scale retrospective studies of archived fixed tumors, a more efficient course would be to use the rosette RP array to identify those SH2 domains that 1.) give strong signals in breast cancer, and 2.) show wide variation in signal intensity when different breast cancer samples are analyzed. As shown above (Task 2), this has now been accomplished.

Another reason for not following up on histochemical-based SH2 profiling of tumor sections was the recent success of a collaborative project with Dr. Peter Nollau of the University Hospital-Eppendorf in Hamburg, Germany. The goal of this project (which is outside the scope of this BCRP funded research) was to develop a high-throughput, multiplexed SH2 profiling platform based on the use of oligonucleotide-tagged SH2 domains, which we term the Oligo-Tagged Multiplex (OTM) approach (Dierck et al., 2006). This novel assay is compatible with both fresh clinical samples and archived formalin-fixed specimens. Because it is a multiplexed assay in which multiple labeled SH2 domains compete for binding in the same reaction, it requires fewer sections and provides better control for relative binding levels of different SH2s than the original histochemical approach proposed for the current study. Therefore we anticipate that in the near future, retrospective studies on archived specimens will be more efficiently accomplished using the new OTM approach.

We still have potential collaborations in place with Dr. David Rimm and Harriet Kluger at Yale University to pursue tissue microarray studies, and Dr. Poornima Hegde has provided invaluable assistance in assembling archived tumor samples for future analysis, and also helping in histological analysis of frozen tumor tissues analyzed by rosette RP assay (Task 2, above).

FIGURES: see Appendix for figures and figure legends

KEY RESEARCH ACCOMPLISHMENTS

2004:

•Development of reproducible, robust reverse-phase macroarray platform for SH2 profiling and its validation using cell lysates

•Generation and purification of ~40 new SH2 domain probes, ~15 of which are suitable for use in SH2 profiling assays
•Establishment of standard conditions for specific binding of SH2 domain probes to fixed tissue sections
•Demonstration in Tissue Microarray experiments that different tumor specimens can have different profiles of SH2 domain binding, validating this approach as a possible method for classifying breast cancers

2005:

• Optimization and validation of robust reverse-phase array platform for SH2 profiling with throughput and reproducibility suitable for high-throughput analysis of clinical tumor samples
• Generation and purification of ~45 new SH2 domain probes in past year
• SH2 profiling of 8 clinical breast cancer samples
• Heirarchical clustering analysis of breast cancer samples based on SH2 binding patterns
• Preliminary correlation of specific SH2 binding (of Eat2 SH2 domain) with presence of inflammation in breast cancer sample

2006:

•Generated probes for virtually all remaining SH2 domains in the human genome (116 of 120 total SH2 domains are now available in the lab)
•Profiled two breast cancer cell lines and 12 cored primary breast tumors using 96 different SH2 domain probes
•Identified SH2 domain probes with high signal-to-noise ratios for analysis of breast cancer cell lines and primary tumor samples
•Performed high-resolution 1-D SH2 domain profiling on 12 cored breast cancer samples to identify binding patterns that can serve as the basis for classification
•Demonstrated that SH2 binding patterns for human breast cancer samples provide novel information that is different from HER2 and EGFR status, and other current standard clinicopathological criteria for classification of breast cancer

REPORTABLE OUTCOMES

2004:

Research Abstract: Kazuya Machida, Christopher M. Thompson, and Bruce J. Mayer. Proteomic profiling of SH2 domain binding. Abstract for platform presentation at the 20th Annual Meeting on Oncogenes, Frederick MD, June 16-20, 2004.

2005:

Research Abstract: Kazuya Machida, Christopher M. Thompson, and Bruce J. Mayer. Profiling human SH2 domains. Abstract for poster presentation at Cold Spring Harbor Meeting on Protein Phosphorylation and Cell Signaling, May18-22, 2005.

Research Abstract: Kazuya Machida, Christopher M. Thompson, and Bruce J. Mayer. Profiling global tyrosine phosphorylation patterns in breast cancer. A novel molecular diagnostic approach. Abstract for poster presentation at the 2005 Era of Hope meeting, June 8-11, 2005.

Review Article: Machida K and Mayer BJ. The SH2 domain: versatile signaling module and pharmaceutical target**.** Biochim Biophys Acta Proteins and Proteomics 2005; 1747:1-25

Invention Disclosure: Bruce J. Mayer, Kazuya Machida, and Peter Nollau, inventors. High-throughput multiplexed SH2 profiling methods. University of Connecticut disclosure #05-926, submitted May 5, 2005.

2006:

Research Abstract: Kazuya Machida, Christopher M. Thompson, and Bruce J. Mayer. Reverse phase phosphotyrosine profiling by SH2 domains. Abstract for poster presentation at the 2006 Gordon Research Conference on Receptor Tyrosine Kinase Signaling, July 16-20, 2006.

Grant Application: Feasibility of SH2 Profiling as a Diagnostic Indicator for Breast Cancer
Foundation: Breast Cancer Alliance, Inc. PI: Kazuya Machida
Submitted: July 31, 2006

Grant Application: Defining Molecular Signatures of Breast Cancer: SH2 Domain-Based Profiling
Foundation: The Susan G. Komen Breast Cancer Foundation PI: Kazuya Machida
Submitted: August 30, 2006

Grant Application: Subclassification of Breast Cancer by SH2 Profiling
Foundation: Connecticut Breast Health Initiative PI: Kazuya Machida
Submitted (executive summary): September 30, 2006

PERSONNEL RECEIVING SALARY FROM THIS PROJECT:

Dr. Kazuya Machida (postdoctoral fellow, Assistant Professor In Residence)

Dr. Poornima Hegde (co-investigator)

Dr. Bruce J. Mayer (Principal Investigator)

CONCLUSIONS

Over the course of this study, we have made tremendous progress in developing a novel molecular diagnostic method that may be used to sub-classify breast cancers based on differences in their tyrosine phosphorylation patterns. When we proposed this study, we knew that different SH2 domains could be used to distinguish different patterns of tyrosine phosphorylation in lysates of tissue culture cells, but we had a number of hurdles before we could use this method to analyze tumor samples. We have now demonstrated that different binding patterns can indeed be discerned in surgical samples from breast cancer patients, and we have developed a quantitative, reliable, high-throughput SH2 profiling method to analyze tumor samples. In our novel rosette RP assay, tens to hundreds of samples can be tested for binding to up to 96 different SH2 domain probes at one time. We have demonstrated that this assay is robust, sensitive, and reproducible. This assay platform now opens the door to quantitative analysis of SH2 binding patterns for breast cancer samples. We have now used this novel system to analyze approximately 20 surgical breat cancer samples. We have shown that SH2 binding profiles provide information beyond that provided by standard clinicopathological staging criteria; thus it is likely that such information could serve as the basis for novel classifications of breast cancer that may be able to better predict clinical outcomes such as response to therapy and recurrence. Establishing correlations between SH2 binding patterns and clinical outcomes will be the focus of our future studies in this area. We also made

some progress in developing a histochemical SH2 binding method that could be used to analyze archived, formalin-fixed tumor samples. Our preliminary studies were moderately encouraging, but revealed potential difficulties in data normalization, throughput, and cost. In future studies, we anticipate using the OTM method (developed outside this project) to perform retrospective analyses of archived specimens to validate the predictive power of SH2 binding profiles.

In summary, the work funded by this project has provided proof-of-principle for the potential usefulness of quantitative SH2 profiling data for tumor classification, and it supported the development of a new and powerful analytic method for profiling phosphorylation patterns in tumors. Although much work remains to be done to validate the usefulness of SH2 profiling in the clinic, it is likely that the approach pioneered in these studies will ultimately provide an important new tool to physicians and breast cancer patients, so they can choose the most effective treatment with the fewest side effects for each patient.

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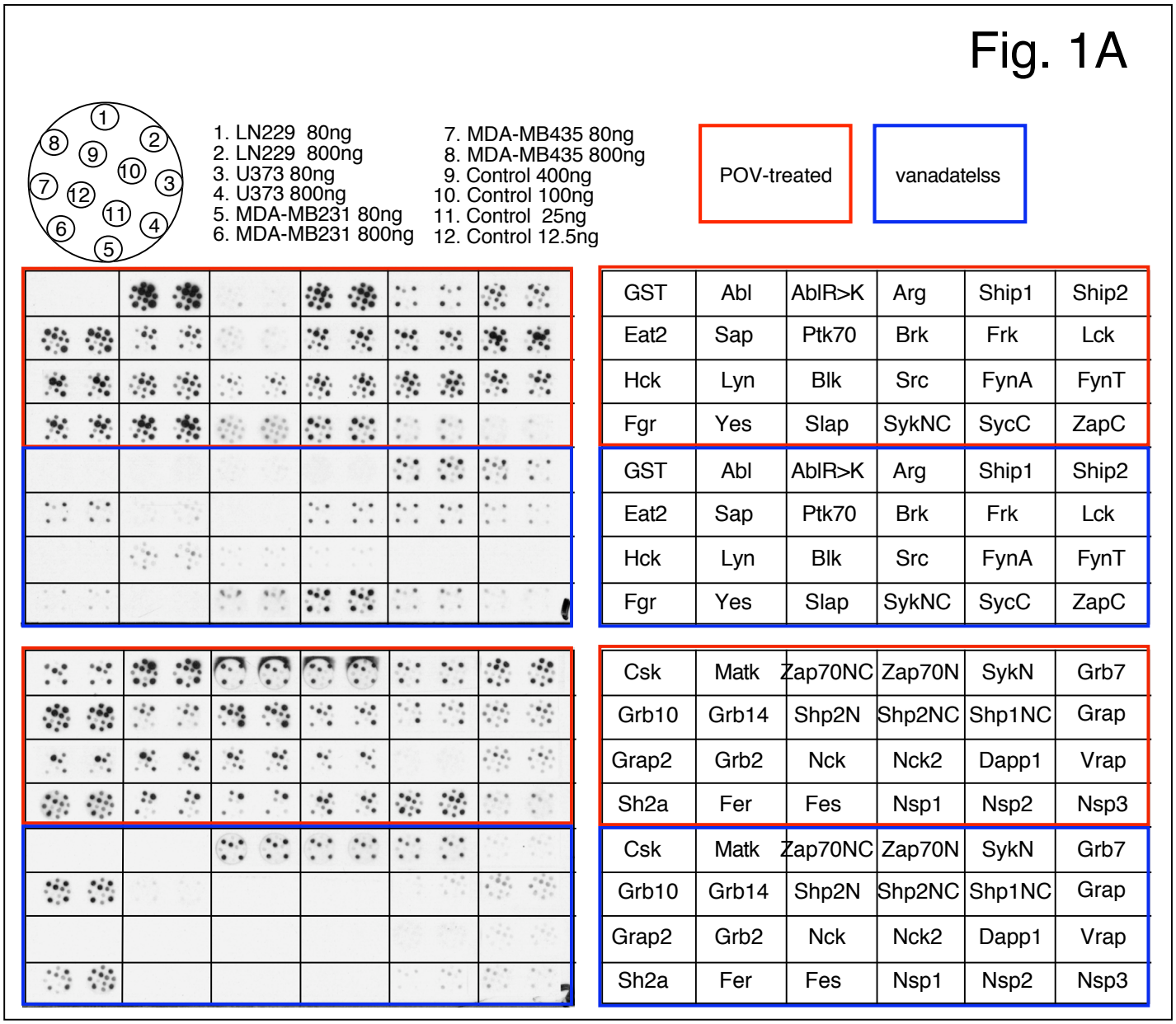
APPENDICES

Figures: Figures 1-5 and associated legends

Research Abstract: Kazuya Machida, Christopher M. Thompson, and Bruce J. Mayer. Reverse phase phosphotyrosine profiling by SH2 domains. Abstract for poster presentation at the 2006 Gordon Research Conference on Receptor Tyrosine Kinase Signaling, July 16-20, 2006.

Figure 1: Rosette RP screening of tumor cell lines using 96 GST-SH2 domain probes. Tumor cell line lysates, or positive control (for POV-treated) or negative control (vanadateless) lysates were spotted as indicated diagrammatically at the top. Samples in red boxes were prepared in the presence of pervanadate (POV-treated) to preserve tyrosine phosphorylation. Samples in blue boxes were prepared in absence of vanadate, leading to dephosphorylation of SH2 binding sites. Arrayed samples were incubated with 96 different SH2 domain probes (as indicated on right), and binding was detected using glutathione-HRP conjugate and ECL (left).



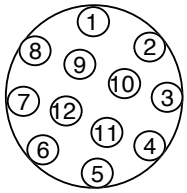
GST	Abl	AblR>K	Arg	Ship1	Ship2
Eat2	Sap	Ptk70	Brk	Frk	Lck
Hck	Lyn	Blk	Src	FynA	FynT
Fgr	Yes	Slap	SykNC	SycC	ZapC

GST	Abl	AblR>K	Arg	Ship1	Ship2
Eat2	Sap	Ptk70	Brk	Frk	Lck
Hck	Lyn	Blk	Src	FynA	FynT
Fgr	Yes	Slap	SykNC	SycC	ZapC

Csk	Matk	Zap70NC	Zap70N	SykN	Grb7
Grb10	Grb14	Shp2N	Shp2NC	Shp1NC	Grap
Grap2	Grb2	Nck	Nck2	Dapp1	Vrap
Sh2a	Fer	Fes	Nsp1	Nsp2	Nsp3

Csk	Matk	Zap70NC	Zap70N	SykN	Grb7
Grb10	Grb14	Shp2N	Shp2NC	Shp1NC	Grap
Grap2	Grb2	Nck	Nck2	Dapp1	Vrap
Sh2a	Fer	Fes	Nsp1	Nsp2	Nsp3

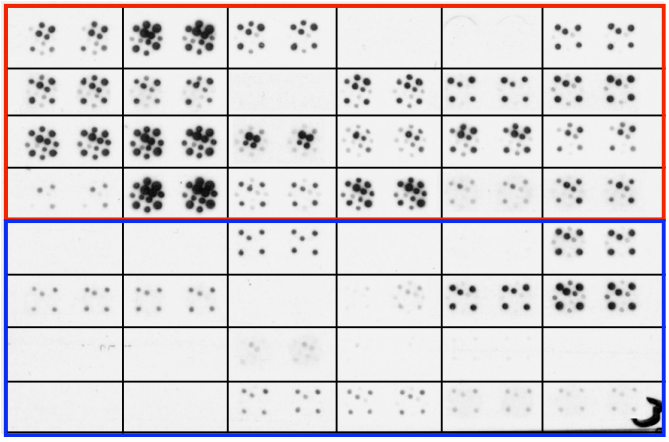
Fig. 1B



1. LN229 80ng
2. LN229 800ng
3. U373 80ng
4. U373 800ng
5. MDA-MB231 80ng
6. MDA-MB231 800ng
7. MDA-MB435 80ng
8. MDA-MB435 800ng
9. Control 400ng
10. Control 100ng
11. Control 25ng
12. Control 12.5ng

POV-treated

vanadatelss



ShcA	Sck	Grb7	ShcC	ShcD	Aps
Lnk	Sh2-b	Sh2-bRK	Shb	Shd	Loc90525
Crk	CrkL	Plcg1NC	Plcg2NC	P85aNC	p55gNC
Vav1	Vav2	Vav3	Tec	Btk	Emt

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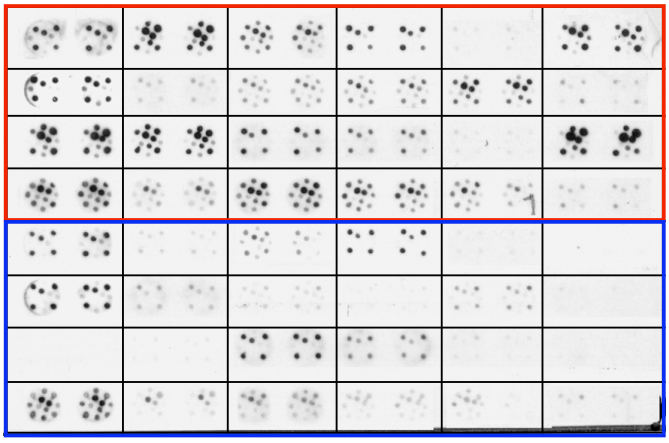
Vav1	Vav2	Vav3	Tec	Btk	Emt



Txk	Bmx	Slp76	Mist	MistRK	GapNC
Chimerin	Chimerin 2	Tensin	Tem6	Tenc1	Cis1
Cten	Nap4	Rin1	RIn2	Rin3	Sh3bp2
CblA	CblB	CblC	Bks	Brdg1	Supt6h

Txk	Bmx	Slp76	Mist	MistRK	GapNC
Chimerin	Chimerin 2	Tensin	Tem6	Tenc1	Cis1
Cten	Nap4	Rin1	RIn2	Rin3	Sh3bp2
CblA	CblB	CblC	Bks	Brdg1	Supt6h

Figure 2. Phosphotyrosine-specific binding of different SH2 domain probes. Control or tumor cell line lysates (same as in Fig. 1) were prepared in the absence or presence of pervanadate in the lysis buffer. In the case of control cell lysates, cultured cells were also briefly incubated with vanadate to increase tyrosine phosphorylation levels before lysis. Rosette RP SH2 profiling assays were performed (see Fig. 1), and total signal for tumor cell lysates and control lysates were quantified by scanning films and analyzing using ImageJ software. The ratio of signal from lysates prepared in the presence of vanadate to that from lysates in the abence of vanadate ('vanadateless") is shown for control cell lines (red bars) and tumor cell lysates (blue bars). A ratio of greater than 1 indicates that the SH2 domain binds more strongly to tyrosine-phosphorylated proteins, as expected for a specific signal. The identity of each of the 96 probes tested is indicated underneath the bars.

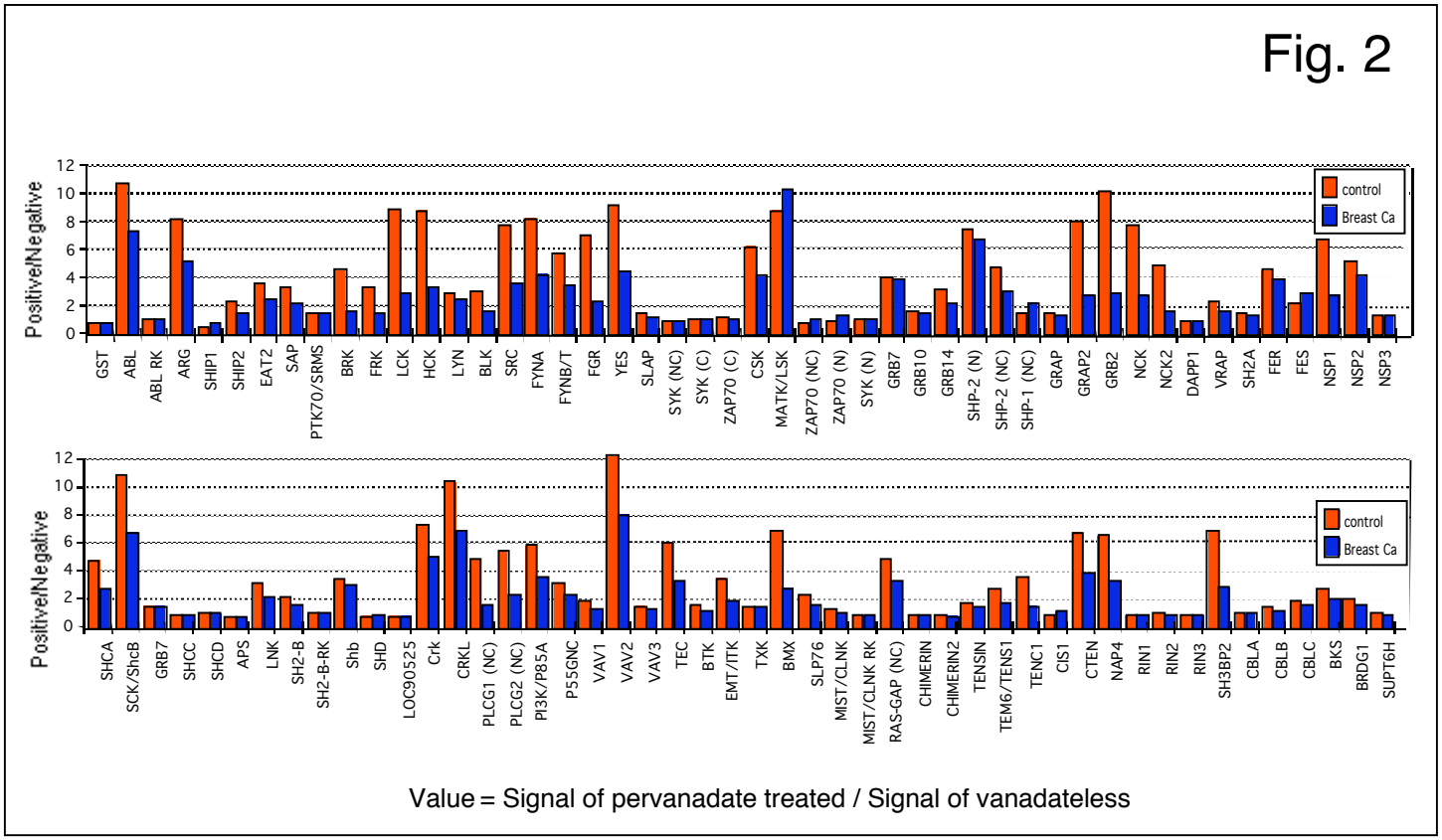


Figure 3: Rosette RP screening of tumor samples 96 GST-SH2 domain probes. Breast cancer tumor lysates, tumor cell line lysates, or positive control (for POV-treated) or negative control (vanadateless) lysates were spotted as indicated diagrammatically at the top (note: samples in red boxes and samples in blue boxes are different, as indicated). Arrayed samples were incubated with 96 different SH2 domain probes (as indicated on right), and binding was detected using glutathione-HRP conjugate and ECL (left). Samples were also incubated with anti-phosphotyrosine antibody (anti-pTyr) to quantify total phosphotyrosine levels, and with anti-tubulin antibody to assess total cell content for each spot, as indicated in upper right panels.

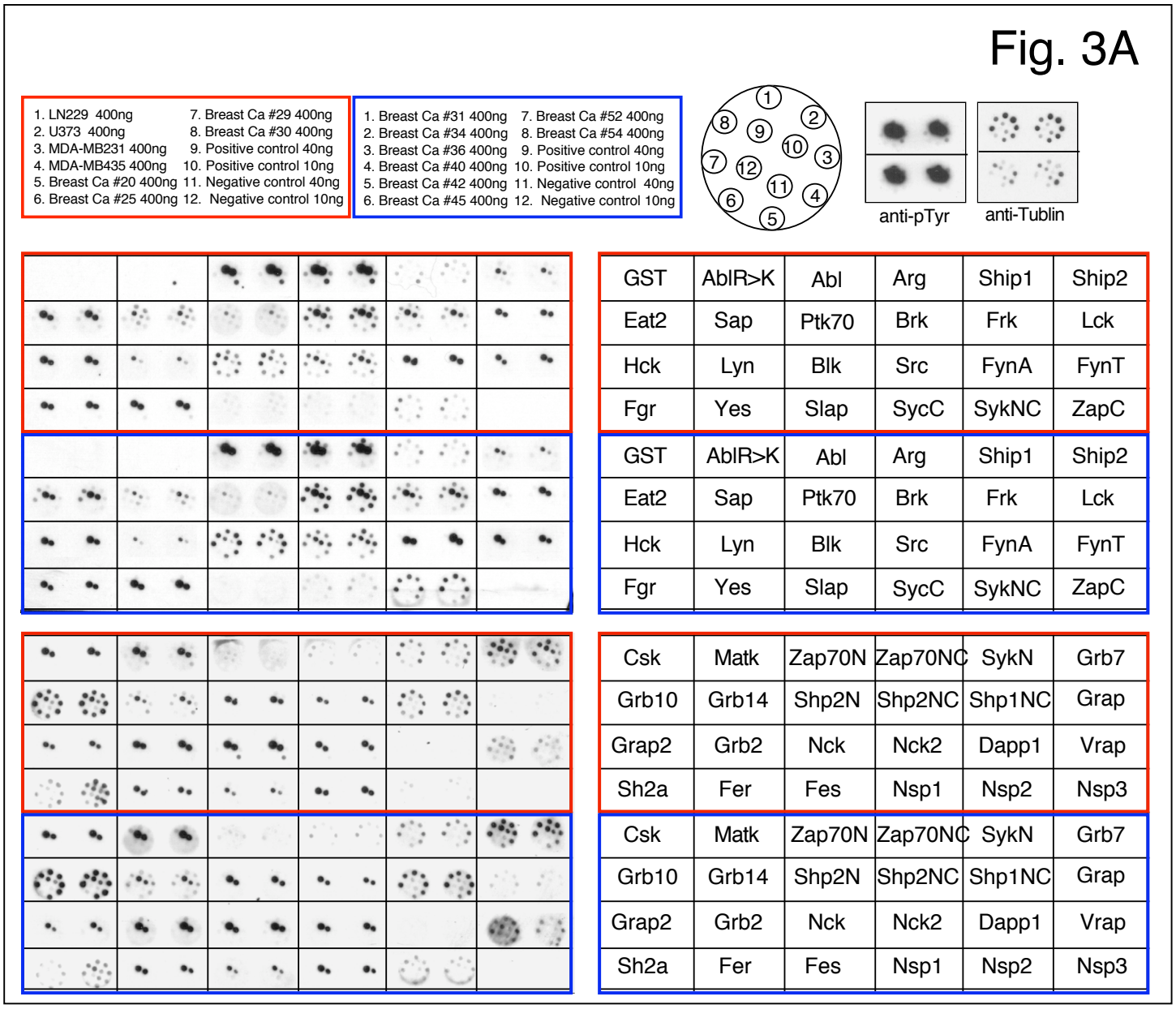
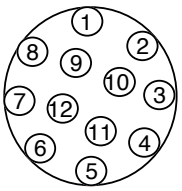
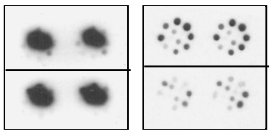


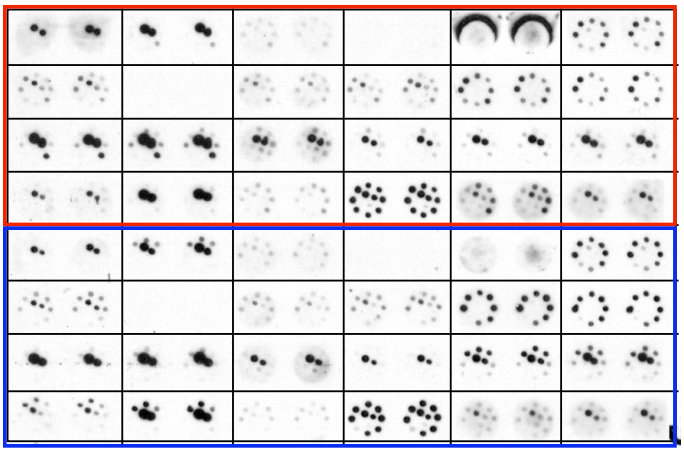
Fig. 3B

1. LN229 400ng
2. U373 400ng
3. MDA-MB231 400ng
4. MDA-MB435 400ng
5. Breast Ca #20 400ng
6. Breast Ca #25 400ng
7. Breast Ca #29 400ng
8. Breast Ca #30 400ng
9. Positive control 40ng
10. Positive control 10ng
11. Negative control 40ng
12. Negative control 10ng

1. Breast Ca #31 400ng
2. Breast Ca #34 400ng
3. Breast Ca #36 400ng
4. Breast Ca #40 400ng
5. Breast Ca #42 400ng
6. Breast Ca #45 400ng
7. Breast Ca #52 400ng
8. Breast Ca #54 400ng
9. Positive control 40ng
10. Positive control 10ng
11. Negative control 40ng
12. Negative control 10ng







ShcA	Sck	PTK70	ShcC	ShcD	Aps
Lnk	Sh2-bRK	Sh2-b	Shb	Shd	Loc90525
Crk	CrkL	Plcg1NC	Plcg2NC	P85aNC	p55gNC
Vav1	Vav2	Vav3	Tec	Btk	Emt

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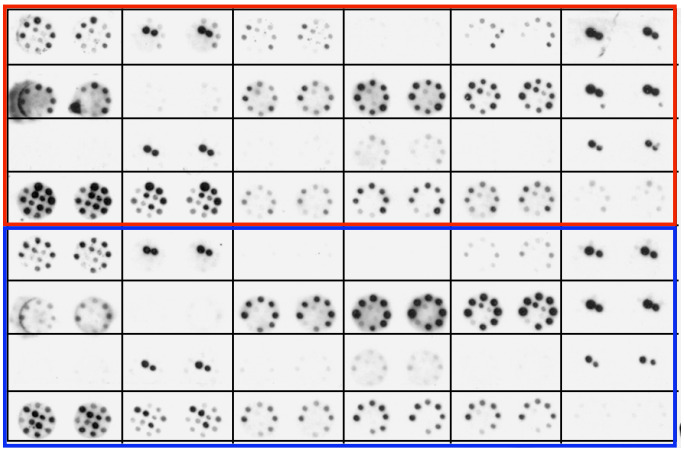
Crk	CrkL	Plcg1NC	Plcg2NC	P85aNC	p55gNC
Vav1	Vav2	Vav3	Tec	Btk	Emt



Txk	Bmx	Slp76	MistRK	Mist	GapNC
Chimerin	Chimerin 2	Tensin	Tem6	Tenc1	Cten
Cis1	Nap4	Rin1	RIn2	Rin3	Sh3bp2
CblA	CblB	CblC	Bks	Brdg1	Supt6h

Txk	Bmx	Slp76	MistRK	Mist	GapNC
Chimerin	Chimerin 2	Tensin	Tem6	Tenc1	Cten
Cis1	Nap4	Rin1	RIn2	Rin3	Sh3bp2
CblA	CblB	CblC	Bks	Brdg1	Supt6h

Figure 4. Phosphotyrosine dependence of SH2 binding to breast cancer samples. 25 SH2 domains were selected based on preliminary screening and tested for phosphotyrosine-dependent binding. Lysates from tumor cell lines, breast cancer samples, or positive and negative controls (prepared in presence or absence of vanadate, respectively) were arrayed on nitrocellulose membranes as indicated on top (note: samples in red boxes and blue boxes are different as indicated). Duplicate filters were prepared, and one filter ("PTP treated") was incubated with purified bacterially expressed PTP-1B to remove phosphotyrosine from proteins. Both filters were incubated with 24 GST-SH2 probes as indicated on right. Binding was quantified, and specific signal (signal from untreated membrane – signal from PTP treated membrane) is shown for each SH2 domain probe in bar graph at bottom. Those GST-SH2 probes giving strong specific signals were selected for analysis by 1D far-Western (see Fig. 5)

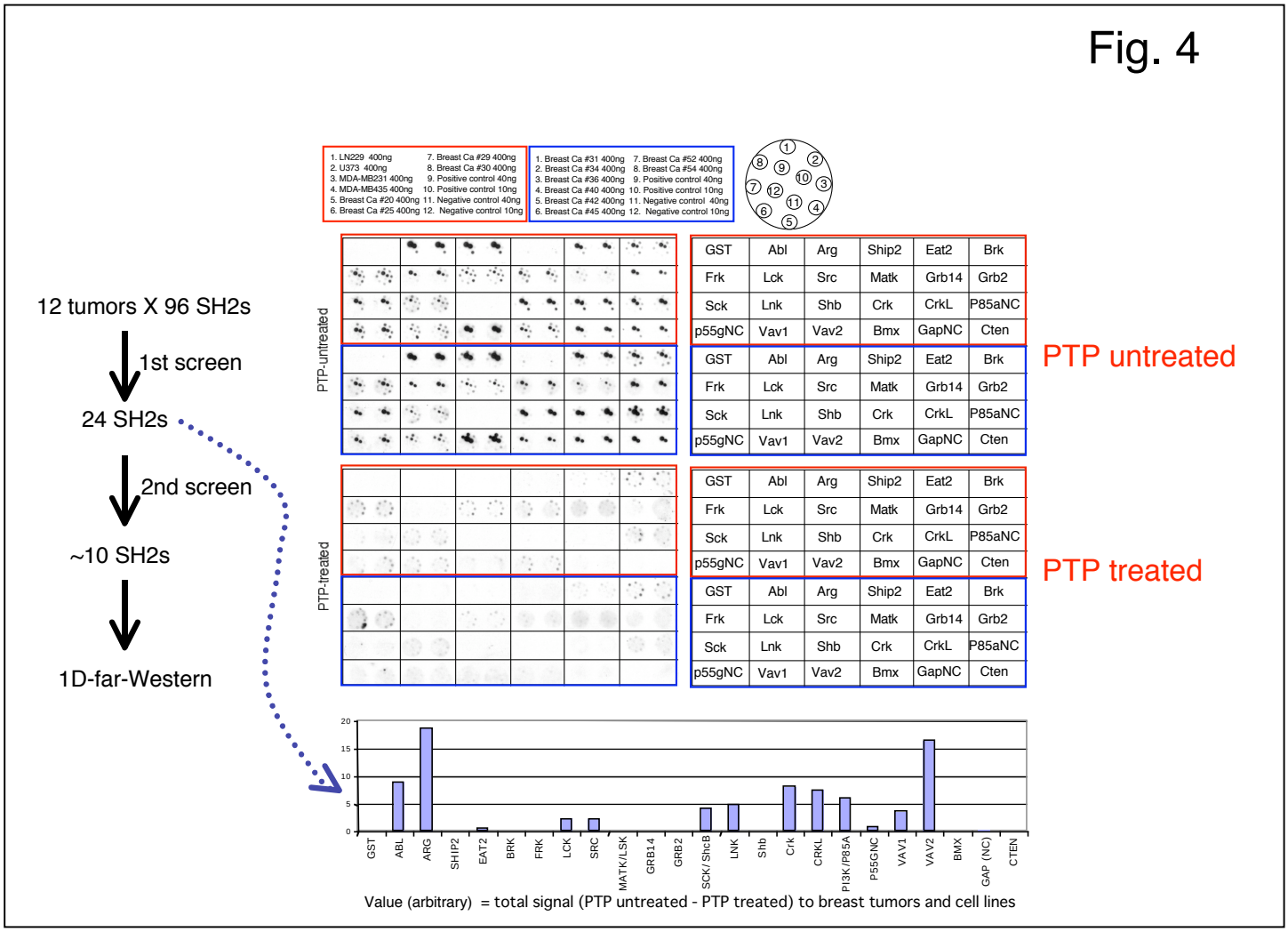
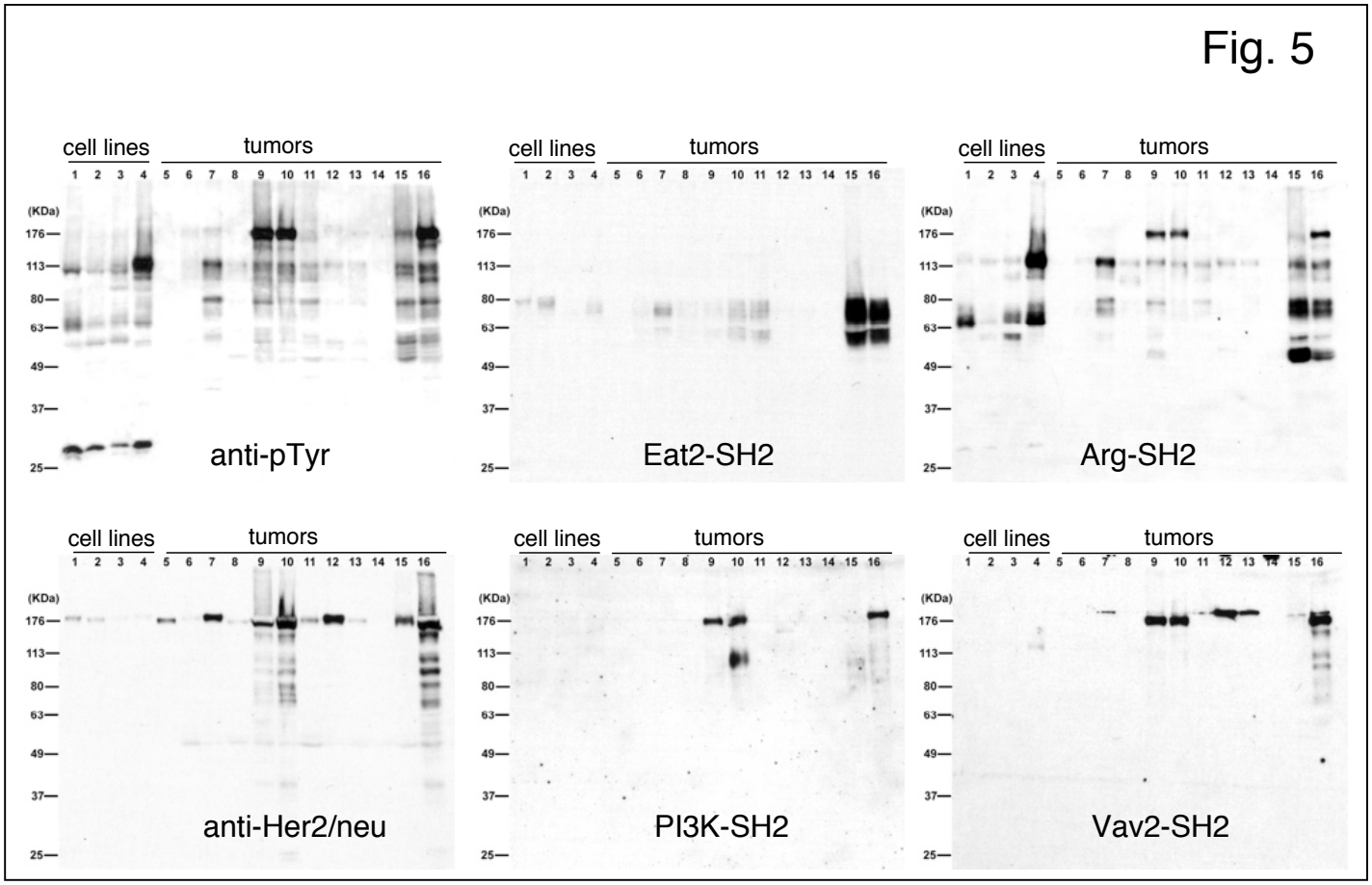


Figure 5: Tyrosine-phosphorylated proteins in tumor cell lines and breast cancer clinical samples. Lysates from cell lines and tumor samples were separated by SDS-PAGE and transferred to nitrocellulose membranes. Duplicate membranes were immunoblotted with antibodies to phosphotyrosine (anti-pTyr) or to Her2/Neu, or were incubated with labeled GST-SH2 domain probes as indicated. After washing, binding was detected by ECL. See Tables 1 and 2 for identity of samples in each lane. Position of molecular weight markers (in kDa) are indicated to the left of each panel.



Research Abstract for poster presentation at the 2006 Gordon Research Conference on Receptor Tyrosine Kinase Signaling, July 16-20, 2006.

Reverse phase phosphotyrosine profiling by SH2 domains

Kazuya Machida, Christopher M. Thompson, and Bruce J. Mayer Department of Genetics and Developmental Biology, University of Connecticut Health Center, Farmington, CT

Tyrosine phosphorylation of proteins can create binding sites for downstream signaling molecules via phosphotyrosine-binding domains such as Src homology 2 (SH2) and phosphotyrosine binding (PTB) domains. Approximately 120 SH2 domains are thought to exist in humans. We have generated GST fusions for almost the entire human complement of SH2 domains, with the aim of using them to profile the global tyrosine phosphorylation state of the cell (termed SH2 profiling). We have established a quantitative multiplexed reverse-phase assay in which large numbers of protein samples (purified proteins or cell lystates) are immobilized on membranes and probed separately with multiple labeled SH2 domains. This proteomic approach permits the rapid and comprehensive analysis of global patterns of SH2 domain binding to specific phosphoproteins, or to all phosphoproteins present in a cell. Using this screening strategy, we have profiled tyrosine phosphorylation changes in response to cell adhesion. SYF cells (deficient for Src, Yes and Fyn) and c-Src-rescued SYF cells were cultured in suspension and adhesion conditions and the levels of tyrosine-phosphorylated binding sites for each SH2 domain were determined. Compared to SYF cells, c-Src-SYF cells showed clearly increased binding for multiple SH2 domains, indicating Src family kinase dependent phosphorylation occurs upon cell adhesion. Subsequent far-Western analysis revealed unique binding patterns for particular SH2 domains. We have identified a number of adhesion-dependent ligands for specific SH2 domains, including p130Cas, Fak, and paxillin. Thus SH2 profiling is a novel tool to reveal the global connectivity of signaling pathways downstream of tyrosine phosphorylation.